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FINAL REPORT

PRODUCTION OF RECOMBINANT FOLLICLE STIMULATING HORMONE (rFSH)

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Production of Recombinant Follicle Stimulating Hormone

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ADF Research Project Final Report

January 7, 2000

(a) Abstract

Commercial game animal production has become a means of diversifying Saskatchewan's agricultural economy. Efficient game farming and export of valuable embryos broaden the economic base for Saskatchewan farmers. Problems encountered in the application of reproductive assisted technologies, such as embryo transfer (ET), to game farmed animals, include marginal reproductive performance due to stress from handling, limiting the profitability of this procedure.

Gonadotropins are hormones that are used in ET for inducing multiple ovulation in domestic animals. Presently gonadotropins are extracted from domestic animal pituitary glands collected at slaughter, which yield crude biochemically undefined products with high variability in bioactivity. The availability of a recombinant gonadotropin with sustained biological activity will provide a tool to minimize stress related to handling and thus improve the success of ET in game farmed species.

The objective of this study was to produce recombinant equine FSH (eFSH), a gonadotropin with sustained biological activity, which can be used in cattle and in game farmed species. The complete coding region of the equine FSH α-subunit gene was amplified by polymerase chain reaction (PCR) as previously reported and the identity confirmed by sequencing. The complete coding region of the equine FSH β-subunit cDNA was reverse transcribed (RT) from equine pituitary RNA and amplified by PCR. Amplified FSH β-cDNA products were confirmed to be 432 base pairs (bp) in length by sequencing five products from separate RT and PCR reactions. The equine FSH β-cDNA and FSH α-gene were subcloned into a mammalian expression vector, pDRSV5.1. Equal amounts of each plasmid were transfected together into dehydrofolate reductase (DHFR) deficient Chinese Hamster Ovary (CHO) cells. RNA from selected clones was harvested and analyzed by Northern blot for FSH subunits gene expression. Fourteen cell lines expressed both the α-subunit gene and β-subunit cDNA. These cell lines secreted the recombinant eFSH protein in the culture media and we are presently in the process of determining its potency in an in vitro assay. We are also in the process of gene amplification, in order to further enhance production of the recombinant eFSH in the culture media. We expect that in a short period of time we will have sufficient amount of recombinant eFSH to conduct studies in cattle, horses and game farmed species.

(b) Executive Summary

The researchers are working towards the development of improved reproductive hormones (gonadotropins) to be used for the production of viable embryos in domestic and game farmed animals. Gonadotropins are hormones that are used in embryo transfer (ET) to induce multiple ovulations (superovulation) in domestic animals. Although ET has been practiced for two decades in cattle, the great variability in both ovulation rates and yield of viable embryos remains a major problem for the animal husbandry industry. Currently, gonadotropins are extracted from porcine pituitaries at slaughter. This procedure yields a poorly defined non-uniform product, believed to be responsible for the great variability observed in ovarian superovulatory response. Recombinant technology circumvents these problems because a uniform product, free of impurities, is produced. Multiple daily injections of gonadotropins are now required for superovulation, which makes this technique expensive and inconvenient in cattle and not suitable for game farmed species. Hormones of equine origin have sustained biological activity, which makes them suitable for inducing superovulation with a single administration, which effectively minimizes handling and the cost of the procedure. The production of equine rFSH will develop a biotechnology industry that will make available a commercial product with superb characteristics, for use in domestic and game farmed animals.

FSH is a product highly demanded by an existing global market. The advantage of rFSH is that there are fewer regulatory barriers to global marketing because of the controlled *in vitro* production, which limits the possibility of disease transmission. Therefore, the immediate commercialization of FSH has great potential for the cattle ET market. In addition, this domestic product will encourage further research in reproduction in cattle and game farmed animals. Maximizing reproductive efficiency and developing new biotechnology aimed at minimizing stress will be essential for the future success and development of new markets for game farmed animals. At the end of the project we expect to have the technology available for the immediate initiation of commercial production of recombinant FSH.

(c) Technical Report

Significant achievements: The production of recombinant equine FSH

Equine FSH β–subunit cDNA cloning and sequencing

There were no published reports of the nucleotide sequence of the equine FSH β-subunit cDNA . The sequence had been predicted from other species and information from the internet was obtained that listed the cDNA sequence of a number of pituitary glycoprotein hormones from a variety of species. The equine FSH β-subunit cDNA was amplified from equine pituitary RNA by reverse transcriptase polymerase chain reactions (RT-PCR) using custom primers. A large number of primers were designed and different reactions mixtures tried. The primers were designed to match highly conserved areas of

the FSH β–cDNA. Primers 1 and 2 (Fig. 1) shown below were used for the amplification of the FSH β–cDNA.

Primer 1: 5'-TCA TAG GCC TTA ATT GTT CGT TCC-3'
Primer 2: 5'-TGT CAG CGT TCT TTA TTC CTT CAT-3'

Figure 1. Oligonucleotide primers used for amplification of equine FSH β–cDNA.

The amplified DNA fragment was 432 bp. This fragment was cloned into the plasmid pCRScript® (GIBCO). The sequence was verified by 3 independent reverse transcriptase and 3 corresponding independent PCR's. We concluded that the sequence of the clone obtained (Fig. 2) was identical to the native sequence of the reported equine FSH β–cDNA subunit.

5'-
ATAGGCCTTAATTGTTCGTTCCAGCCCAAGATGAAGTCAGTCCAGTTTTGT
TTCCTTTTCTGTTGCTGGAAAGCAGTCTGCTGCAATAGCTGTGAGCTGACC
AACATCACCATCGCCGTGGAGAAGGAGGAATGTGGCTTCTGCATAAGCAT
CAACACCACCTGGTGTGCGGGCTACTGCTACACCCGGGACCTGGTGTACA
AGGACCCAGCCCGGCCCAACATCCAGAAAACATGCACCTTCAAGGAGCTG
GTGTACGAGACAGTGAAAGTGCCTGGCTGTGCTCACCACGCGGACTCCCT
GTACACGTACCCGGTGGCCACTGCATGTCACTGTGGCAAATGTAACAGCG
ACAGCACTGACTGCACCGTGCGAGGTCTGGGGCCCAGCTACTGCTCCTTCG
GTGACATGAAGGAATAAAGAACGCTGAC-3'

Figure 2. Equine FSH β–cDNA (432 bp)

The equine FSH-β cDNA (432 bp) was subcloned into the pDRSV-5.1 expression vector, supplied by Dr. Gary Sherman (Fig. 3).

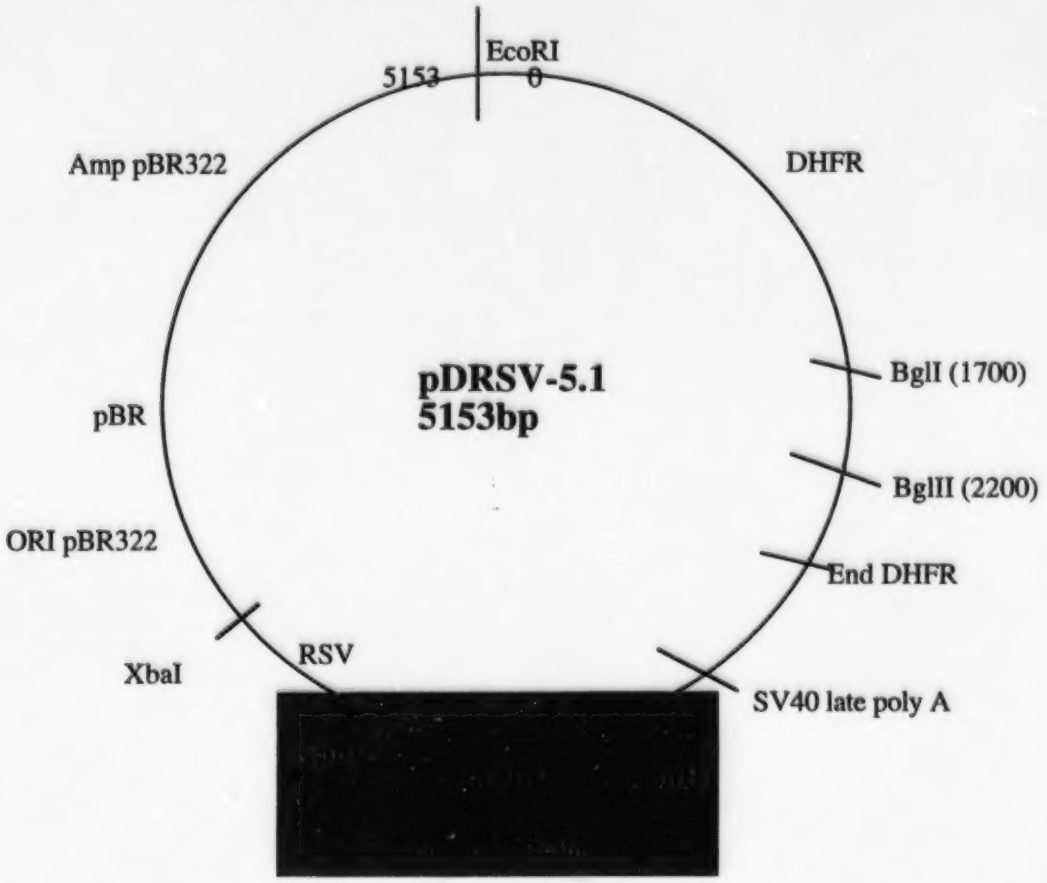

Figure 3. Plasmid pDRSV-5.1

Subcloning Strategy

The insert was restriction digested out of the pCRScript plasmid with KpnI and XhoI. The pDRSV-5.1 plasmid was restriction digested with KpnI and BamHI. T4 DNA ligase was used for sticky ligation of the equine FSH _ cDNA fragment into the pCRScript. XhoI and BamHI ends were filled-in with the Klenow fragment of DNA polymerase in the presence of dNTP's. A blunt ligation using T4 DNA ligase joined the filled in ends of XhoI and BamHI.

The insert was then re-sequenced to confirm that it was not altered during the cloning process. The equine FSH α–subunit gene was amplified by Dr. Sherman using primers 3 and 4 (Fig 4), which was cloned into the pDRSV-5.1 expression vector.

Primer 3: 5'- AAGAGCCATGGATTACTACAGA-3'
Primer 4: 5'-TCAGCAGTCATCCACAAGGC-3'

Figure 4. Oligonucleotide primers used for amplification of equine FSH-α cDNA.

5'-
AAGAGCCATGGATTACTACAGAAAACATGCAGCTGTCATCCTGGCCACAT
TGTC

exon II
CGTGTTTCTCGATATTCTCCATTCCTTTCCTGATGGAGAGTTTACAACGCA
GGAT
exon III
TGCCCAGAATGCAAGCTAAGGGAAAACAAGTACTTCTTCAAACTGGGCGT
CCC

GATTTACCAGTGTAAGGGCTGCTGCTTCTCCAGAGCGTACCCCACTCCAGC
AA

GGTCCAGGAAGACAATGTTGGTCCCAAAGAACATCACCTCAGAATCCACA
TGC

exon III exon IV
TGTGTGGCCAAAGCATTTATCAGGGTCACAGTGATGGGAAACATCAAGTT
GGA

GAACCACACCCAGTGCTATTGCAGCACTTGCTATCACCACAAGATTTAAA
GGTTT

CACCAAGTGCCTTGTGGATGACTGCTGATTTCCTGGAGTGGAAAATTAAT
TTG

TTCAATGTGTATGGCCTTGTGAGATACCCCCCACCTTTCTCTTACCATTCCC
CTT

TGGCATGCTTCGAGGATATACTGCAGCTTTGTTGTCTTTCTCTTTATCTGA
CAG

TCTGATCGGCAGTCTAGCTCTTTTGATTTGGAATGAAATACAGCATTTTAG
CATG

ATCACAAAAAGCTGGTTCCACAGGAAATAAAGTCTTTTAAAATCATCA-3'

Figure 5. Equine FSH α-subunit cDNA (2200-2300bp). Only exon sequences are represented.

Chinese hamster ovarian cells deficient in the enzyme dihydrofolate reductase (DHFR), CHO 9096 were transformed with the construct containing the FSH α-subunit gene and β–subunit cDNA. A common feature of the cloning vectors used is that they also express the DHFR gene, which is a selection marker. Thus, any cells that survived in selective media (MEM-α without ribonucleotides and deoxyribonucleotides, with dialyzed fetal bovine serum) contained at least one of the vectors. We obtained approximately 180 individual clones, which potentially contained one or both of the subunits. Northern blot analysis was used to confirm the expression of equine FSH and to identify clones with expression of α- and β-subunits. Forty clones were screened by a radioimmunoassay specific for the equine FSH dimer (α– and β-subunits). This assay was performed by Dr. Albert Parlow, Scientific Director, National Hormone & Pituitary Program, Harbor-UCLA Medical Center, Torrance, Ca, USA. Initially Dr. Parlow did not detect FSH immunoactivity in the samples. However, in subsequent assays, Dr. Parlow increased the volume of the sample and detected levels of FSH immunoactivity in all samples (see Dr. Parlow's report). Therefore we successfully obtained cell clones, but the level of FSH was low.

We continued to concentrate our efforts towards increasing the expression of FSH through a process of gene amplification. The strategy of gene amplification is based on inhibition of the DHFR by the drug methotrexate, which kills cells expressing low levels of the enzyme. Thus, cells expressing high levels of the enzyme, with higher copy numbers of the gene, survive the treatment. Therefore, by treating transformed cells with increasing amounts of methotrexate it is possible to increase the number of copies of the FSH α- and β-subunit genes and thus, increase the amount of FSH into the culture media. Early in the process of gene amplification we selected clones which were cultured at very low concentrations (0.005 μM) of methotrexate. Over the past four months cells were subsequently grown in 0.02 and 0.05 μM methotrexate. Currently we have cells growing in 0.02, 0.05 and 0.5 μM methotrexate. When a sufficient amount of cells are grown in 0.5 μM methotrexate, we will conduct Northern blot analysis to test for levels of expression of the FSH α– and β-subunit genes. We are also collecting culture media for a new eFSH assay. In any case, amplification by increasing the level of methotrexate in the medium will continue until cells are growing in 20 to 80 μM methotrexate. At this concentration of methotrexate cells should contain 500 to 2000 copies of the transfected FSH α– and β-subunit genes. We estimate that this level of amplification can be achieved in the next four to five months, at which time cells will produce sufficient amounts of eFSH to be used in animal tests.

We are also selecting clones using Northern blot analysis for a balanced production of both subunits because cells with balanced numbers of subunits are more efficient in their production of FSH glycoprotein. We have also continued with an alternative strategy for cloning the gonadotropin subunits. We successfully cloned the equine β-subunit gene into a more advanced mammalian expression vector, recently developed by our collaborator Dr. Gary Sherman, from the University of Nebraska. This is a dual site

vector, designed for expressing the genes of heterodimeric hormones, such as FSH. We are currently at the initial stage of transfecting CHO cells with this vector. The advantage of this strategy is that a balanced expression of the inserted genes can be obtained in transfected cells and should maximize the efficiency of rFSH, since both genes are introduced at the same time. A balanced expression of the two subunits is important to optimize rFSH secretion.

Future

The PI is very enthused with the experience acquired during the granted period. We are now in an excellent position and committed to continue developing this project. Therefore, we recently submitted an application for renewal of our ADF grant, in which we propose to produce a homologous recombinant elk FSH. Producing game farmed species-specific gonadotropins will an enourmous advantage for the Saskatchewan producers and for the diversification of the provincial economy.

Plans for Commercialization

We are confident that, pending finalization of the gene amplification process we will obtain sufficient amounts of hormone for commercial purposes. We have been contacted by Dr. Bin Yan, Technology Transfer Manager, for the University of Saskatchewan Technologies Inc. Dr. Yan expressed an interest in commercializing the recombinant equine FSH.

Following the presentation of our results at the Annual Meeting of the Society for the Study of Reproduction in Pullman, WA, August 1999, the PI was approached by Dr. Marc-Andre Sirard, from the Centre de Recherche in Biologie de la Reproduction, Laval University. Dr. Sirard offered collaboration using a transgenic porcine model developed in his institution to express the FSH genes cloned in our laboratory. The transgenic porcine model offers an alternative system to the CHO cells, which will result in an economic production of recombinant hormones. We are very enthusiastic about this collaboration which will result in a reduced cost and dramatically improve the marketability of the recombinant hormone. This opportunity will also open an important avenue of interaction with the porcine industry in the province of Saskatchewan. A copy of Dr. Sirard's letter is appended in this report. We also have a manuscript in preparation for submission to the Journal *Theriogenology*.

(d) Personnel

Unfortunately Dr. Rodway has left our group and is actively seeking a faculty position. While we offer her our best wishes, we will miss her commitment and dedication. We have hired a graduate student enrolled in a Master of Science degree program, Mr. Michael Furlan, who will work full time in the recombinant FSH project. Also, we are currently in the process of hiring a technician trained in molecular biology techniques to work with Mr Furlan on this project.

(e) Equipment

Currently no project funds have been required to purchase equipment for items costing $500.00 or more.

(f) Project Developed Materials

Not applicable at this time

(g) Project Photos

Not applicable at this time

(h) Acknowlegement

The PI gratefully acknowledges that Dr. Marie Rodway's salary was paid by the School of Medicine, University of Saskatchewan. The last two months of Dr. Rodway's salary was paid from the ADF grant account.

(i) Expense Statement

The statement of project receipts and expenditures will be forwarded by the University of Saskatchewan, office of Research Accounts.

A. F. PARLOW
NATIONAL HORMONE & PITUITARY PROGRAM

-FSH in CHEDRESE EQUINE MEDIA SAMPLES REC'D 6-30-99

	* In terms of eFSH AFP5022B	
MEDIA SAMPLE	eFSH * (ng/ml)	95% LIMITS
RODWAY #1	6	5 - 8
RODWAY #2	24	19 - 32
RODWAY #3	205	171 - 249
RODWAY #4	2	1 - 3
RODWAY #5	12	9 - 16

eFSH RIA #30 on 1-JUL-99

* In terms of eFSH AFP5022B

IOD Ag = Equine FSH AFP5022B

ANTISERUM = Anti-pFSH AFP2062096 (Rb)

FINAL DILUTION = 1:50,000

B0/T = 33.8%

Centre de Recherche en
Biologie de la Reproduction

Québec, 30/8/99

Dear Jorge:

I was impressed by your poster presentation at the SSR meeting in Pullman, thisyear. Your advances in the cloning and expression of the equine gonadotropins are very interesting.

We have recently developed a system using transgenic pigs to express recombinant genes in the accessory sexual glands (Patent WO9845419A1: TRANSGENIC EXPRESSION IN GENITAL TRACT AND SEXUAL ACCESSORY GLANDS. We believe that this system would be very well suited for expressing gonadotropins. This system has several advantages that could benefit your project. Production of recombinant proteins in the accessory glands of pigs is a more cost-effective method than culture of transformed Chinese hamster ovarian cells. It is also expected that a glycoprotein expressed in pigs would be of higher biological activity since the patter of glycosylation is likely to be better in a live animal and in a relatively closely related species.

We are actually considering the production of recombinant FSH from other species in conjunction with a Canadian firm involved in the distribution of FSH. We suggest that you consider the transgenic pig to develop your product for commercial application and we would be interested in a collaboration with you towards that goal.

Sincerely,

Marc-André Sirard DMV, PhD
Professeur, titulaire de la chaire Claudes Hayes
Directeur du Centre de Recherche en Biologie de la Reproduction

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